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Histones as a Target for Influenza Virus Matrix Protein M1

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Matrix protein M1 purified from influenza A and B viruses has been analyzed for its ability to specifically interact with cellular proteins by immune coprecipitation and by an *in vitro* binding assay on nitrocellulose on PVDF membranes. When M1 was mixed with lysates of uninfected cells there was selective binding of histones H2A, H2B, H3, and H4. Week binding of H1 was also observed. The binding specificity of M1 was confirmed by using purified histones. The M1–histone complexes were dependent on pH and ionic strength, indicating electrostatic interactions. Chemical cleavage of M1 by formic acid into an N-terminal 9-kDa fragment and a C-terminal 18-kDa fragment did not abolish interaction with histones. However, after treatment with 1 *M* sodium chloride cleaved M1 no longer bound to histones, whereas uncleaved M1 showed an increased binding activity after salt treatment. These findings suggest that both N- and C-terminal domains of M1 are involved in histone binding and that conformation of M is an important factor in this interaction. The data support the notion that there is specific interaction of M1 with nucleosomes during the nuclear phase of influenza virus replication.

INTRODUCTION

Matrix protein M1 (~27 kDa) is a major structural protein of influenza virus, with about 3000 molecules per virion (Schulze, 1972). It is highly conserved among influenza viruses and consists of 252 and 248 amino acid residues with types A and B, respectively (Briedis *et al.,* 1982). The hydrophobic polypeptide binds to membranes (Kretzschmar *et al.,* 1996; Zhang and Lamb, 1996) and is thought to interact with the lipid bilayer in the virion via its hydrophobic sequences (Gregoriades and Frangione, 1981; Ye *et al.,* 1987). M1 also has basic amino acid clusters and a zinc-finger motif (Wakefield and Brownlee, 1989; Winter and Fields, 1980) as observed in DNA/RNA-binding proteins (Coleman, 1992). Because of these charged clusters M1 is likely to interact with viral ribonucleoprotein (RNP) by ionic links (Melnikov *et al.,* 1985; Wakefield and Brownlee, 1989; Ye *et al.,* 1989). In virions, the 14-kDa NS2 polypeptide (130–200 molecules per virion) was also found to be associated with M1 (Ward *et al.,* 1995; Yasuda *et al.,* 1993).

The available evidence indicates that M1 has several functions in virus replication. First M1 seems to dissociate from the incoming virus RNP during the uncoating process (Martin and Helenius, 1991a; Zhirnov, 1990), thereby allowing transport of the RNP complex into the nucleus (Bui *et al.,* 1996) the site of viral transcription and replication (Herz *et al.,* 1981; Jackson *et al.,* 1982). Subsequently, newly synthesized M1 enters the nucleus and is then exported into the cytoplasm and transported to the plasma membrane for assembly into mature virions (Martin and Helenius, 1991b; Rey and Nayak, 1992). Transport into the nucleus appears to be mediated by the nuclear localization signal RKLKR (a.a. 101–105) (Ye *et al.,* 1995). The nuclear shuttle transport of M1 was shown to be a pH-dependent event (Bui *et al.,* 1996) and to be linked to the nucleocytoplasmic shuttling of viral RNP (Martin and Helenius, 1991b; Whittaker *et al.,* 1995, 1996).

To understand the intracellular functions of M1 it is important to find out whether there are specific interactions with host cell proteins. The experiments reported here were undertaken to indentify such proteins. For this purpose we have studied the binding of M1 to electrophoretically fractionated proteins of canine kidney (MDCK), monkey kidney (CV-1), and chicken fibroblast (CEF) cells and to purified histones by a highly sensitive affinity-binding assay on nitrocellulose and PVDF membranes and cross-precipitation analysis of M1–histone complexes with anti-M1-specific antibodies. It is shown that M1 isolated from virions by acidic extraction (Zhirnov, 1992) selectively interacts with histones H2A, H2B, H3, and H4, and more weakly with H1, and that these interactions depend on the conformational state of M1. The data suggest that interaction of M1 with chromatin is an important step in the nuclear phase of influenza virus replication.

MATERIALS AND METHODS

Viruses and cells

Influenza viruses A/WSN/33 (H1N1), A/FPV/Rostock/34 (H7N7), A/Aichi/2/68 (H3N2), and B/Hong Kong/72 were

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propagated in 11-day old embryonated chicken eggs (Zhirnov *et al.,* 1985). Primary cultures of chick embryo fibroblasts (CEF), MDCK cells (subline 2) (Fuller *et al.,* 1984), MDCK cells (standard line), and CV-1 cells were grown in Dulbecco's medium containing 5% fetal bovine serum (Gibco BRL). Cell cultures were infected at a m.o.i. of about 5 PFU/cell.

Cell fractionation

Uninfected and infected MDCK, CV-1, and CEF cultures (ca. 10^6 cells) were suspended in 1 ml of PBS containing 1.5% NP-40 and centrifuged at 2000 *g.* The supernatant (cytoplasm) was removed, and the pellet was suspended in 5 ml of PBS containing 1% NP-40 and sedimented at 2500 rpm for 20 min through 4 ml of 25% sucrose containing 150 m*M* NaCl, 5 m*M* Tris–HCl (pH 7.4), 0.5% NP-40, and 50 TIU/ml aprotinin. This procedure was repeated thrice and the final pellet (nuclei) was dissolved in PBS and sonicated to reduce viscosity. Cell homogenate, cytoplasm, and nuclei were then dissolved in SDS–DTT solution and analyzed by SDS–PAGE.

Purification and cleavage of M1 protein

The method of M1 purification was described in detail earlier (Zhirnov, 1992). Briefly, virus-containing allantoic fluid was clarified and then pelleted through 25% glycerol, containing 150 m*M* NaCl, 2 m*M* Tris–Mes (2(*N*-morpholino)ethansulfonic acid) (pH 7.4), 50 TIU/ml aprotinin, 2% non-ionic detergent NP-40 (SW 27 rotor; 22,000 rpm for 2.5 hr). The pellet (virion cores) was suspended in buffer, containing 20 m*M* Mes (pH 4.5) and 100 m*M* NaCl, and centrifuged at 12,000 *g* for 10 min. The supernatant (purified M1) was withdrawn. For binding assays M1 prepared by the above method was dissolved in acidic medium containing 20 m*M* Mes (pH 4.5). In order to change the M1 solution to neutral or alkaline pH and to prevent the autoaggregation of M1 by the pH shift, the following procedure was used. After acidic extraction M1 was diluted in 2% BSA (~10,000-fold excess of BSA compared to the concentration of M1) containing 100 m*M* Tris–Mes with the desirable pH value. M1 remained in the supernatant after high-speed centrifugation at 120,000 *g.*

For chemical cleavage, an aliquot of the solution of purified M1 (~120 μg of protein) was mixed with 3 vol of 99% formic acid (Merck)) and incubated at 37° for 96–112 hr. After incubation the M1 hydrolysate was diluted 10 times with water and then concentrated 10-fold in a Centricon-3000 concentrator (Amicon). The concentrate was frozen and lyophylized in a Speed-VAC and finally dissolved in deionized water to a final concentration of M1 of ~0.5 mg/ml.

Membrane binding assay

After SDS–PAGE the polypeptides were transferred from the gel onto nitrocellulose or 0.45-μm PVDF membranes (Millipore) by semidried electroblotting with Tris–HCl–ε-aminocaproic acid buffer (pH 9.0) (Kyhse-Anderson, 1984). Membranes were washed with 150 m*M* phosphate-buffered saline (PBS) and incubated overnight at 4° in 10% dried milk prepared in PBS. After washing with PBS, membranes were incubated for 1 hr at 37° in buffer, containing 2% BSA, 100 m*M* NaCl, 100 m*M* Tris–HCl (pH 8.4), 0.001% NP-40, and additionally 1.0–0.4 μg/ml of the purified M1 protein. After incubation, the membranes were exposed successively to either anti-M1-monospecific rabbit serum or anti-M1 mouse monoclonal antibodies (Mab), biotin-conjugated secondary antibodies, and streptavidin–peroxidase complex (Amersham) followed by visualization of M1-positive bands with the Amersham enhanced chemiluminescence (ECL) procedure using Kodak XAR film.

Immune coprecipitation analysis

Ten microliters of purified M1 (~5 μg of protein) was added to 200 μl of an equimolar mixture of all five histones (~20 μg of protein; Boehringer) prepared in 1% BSA, 25 TIU/ml of aprotinin, 50 m*M* Tris–Mes (pH 7.4), and 200 m*M* NaCl. In salt pretreatment experiments the M1 solution contained either 1 or 2 *M* NaCl, and the concentration of NaCl in the histone solution was reduced to obtain a final NaCl concentration of 200 m*M.* This mixture was incubated for 1 hr at 37°, and 4 μl of anti-M1 Mab clone M2-1C6 was added followed by additional incubation at 20° for 1 hr. One hundred microliters of 50% protein A–Sepharose (Sigma) presaturated with anti-mouse affinity-purified rabbit Ig (DAKO) was then added and the mixture was incubated at 20° for 45 min. Following binding of M1 and M1–histone complex to protein A via Mab M2-1C6 and anti-mouse Ig, protein A–Sepharose was washed four times with buffer containing 200 m*M* NaCl, 25 m*M* Tris–Mes (pH 7.4), 0.2% Triton X-100, 0.1% Tween 20. The absorbed polypeptides were dissolved in 1% SDS, 10 m*M* DTT and separated by SDS–PAGE. Histones and M1 were visualized by Western blot–ECL using sheep anti-histone ($H3^+$ serum; Biogenesis) and goat anti-M1 (Biogenesis) sera and HRP-conjugated rabbit anti-sheep and anti-goat Ig (DAKO), respectively.

Antibodies

The mouse anti-M1 monoclonal antibody clones M2-1C6, 5C-9, and 7E-5 described earlier (Yasuda *et al.,* 1993) and clone GA2b (Serotec) were used. Sheep serum $H3^+$ against all histones (Biogenesis), goat monospecific anti-M1 Ig, (Biogenesis), monospecific rabbit antiserum prepared against M1 of A/FPV/Rostock/34 virus, and a mouse serum prepared against influenza B/HK/72 were also used in this study.

Polyacrylamide gel electrophoresis (PAGE)

Polypeptides were electrophoresed on either 12 or 13.5% polyacrylamide gels containing SDS, as described

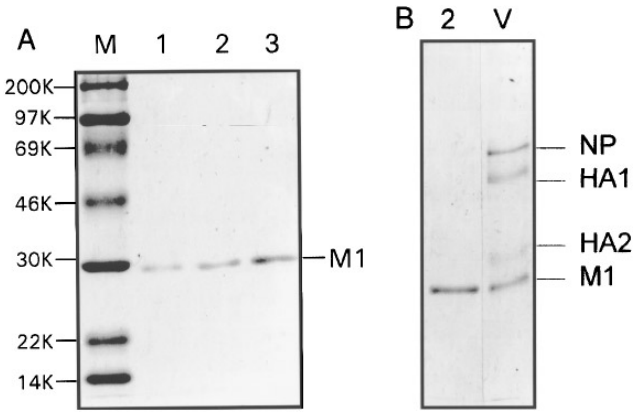

FIG. 1. Electrophoretic analysis of influenza virus matrix protein M1 isolated from virions by acidic extraction. Lane M: marker polypeptides. Lane V: virion polypeptides. Lanes 1, 2, 3, purified M1 protein of A/FPV (A) and B/HK (B), 0.5, 1.2, 2.5 μg, respectively.

earlier (Zhirnov and Grigoriev, 1994). For protein staining the protocol recommended by Pharmacia with Coomassie blue R-350 was applied. The following polypeptide marker kit (Amersham) was used: 200 kDa, myosin; 97.4 kDa, phosphorylase b; 69 kDa, bovine serum albumin; 46 kDa, ovalbumin; 30 kDa, carbonic anhydrase; 21.5 kDa, trypsin inhibitor; 14.3 kDa, lysozyme.

RESULTS

The soluble form of the M1 protein was obtained by acidic extraction from influenza viruses A/WSN/33 (H1N1), A/Aichi/2/68 (H3N2), A/chicken/Germany/34 (A/FPV/Rostock/34) (H7N7), A/X-31 (H3N2), and B/Hong Kong/5/72. M1 purified by this procedure was predominantly in monomeric and possibly dimeric forms and retained its biological activity, such as inhibition of the transcriptase activity of viral RNP (Zhirnov, 1992). The purity of isolated M1 was higher than 90%, as indicated by stained polyacrylamide gels (Fig. 1). In order to determine host cell or viral proteins recognized by M1, we carried out Western blot analysis. Homogenates of MDCK cells, CV-1 cells, and CEF were electrophoresed (Fig. 2A) and transferred to membranes, which were subsequently exposed to M1 followed by decoration with either monoclonal or monospecific anti-M1 antibodies and a second antibody conjugated with biotin or horseradish peroxidase (HRP). A typical M1-binding experiment is shown in Fig. 2B. It can be seen that M1 of the A/Aichi/68 virus bound selectively to two classes of cellular polypeptides with molecular weights of about 32 and 14 kDa (Fig. 2B, middle panel). Analogous results were obtained with M1 isolated from A/WSN/33, A/X-31, A/FPV/Rostock/34, and B/HK/72 viruses (data not shown). High-molecular-weight components hp1 (~150 kDa) and hp2 (~90 kDa) displayed a marked ECL signal independently of the presence of M1 (Fig. 2B, left panel) and anti-M1 antibodies (Fig. 2B, right panel) in the incubation buffer. Therefore, these bands were interpreted as endogenous host cell proteins displaying peroxidase ECL inducing (or streptavidin binding) activities.

The question arose whether the cellular 32- and 14-kDa polypeptides were cytoplasmic or nuclear components. For this purpose, cells were fractionated into cytoplasmic and nuclear fractions (Fig. 3A), and their proteins were assayed for M1 binding (Fig. 3B). As can be seen in Fig. 3B, both 32- and 14-kDa proteins were recognized by M1 in the nuclear fraction. The 14-kDa host cell polypeptides were found to be major bands on the Coomassie-stained gel of the nuclear fraction, indicating that they are major structural elements of the nucleus (Fig. 3A). Size and nuclear location of the 32- and 14-kDa proteins implied that they are histones.

To test this hypothesis purified histones H1, H2A, H2B, H3, and H4 were used in the M1-binding assay. As shown in Fig. 4, M1 of the FPV strain bound to purified histones H1, H2A, H2B, H3, and H4. Interestingly, M1 binding to histone proteins H2A, H2B, H3, and H4 was similar, whereas the interaction of M1 with H1 was weaker, as indicated by the higher concentrations of M1 required in the incubation medium for H1 recognition (Fig. 4A). Similar results were obtained with M1 of influenza virus strains A/WSN and B/HK. These data strengthen the concept that the influenza matrix protein M1 has a specific affinity to histones.

In the next experiments we have studied characteristics of the dependence of the M1–histone interaction on ionic strength, pH, Ca^{2+}, and Mg^{2+} ions, and SH-reducing agents. The M1–histone interaction was found to decrease dramatically at 450 m*M* NaCl (Fig. 4B). M1–histone binding was not affected by the Ca^{2+} and Mg^{2+} ions and by EDTA (data not shown). A special point of investigation was whether disulfide links are involved in M1 binding. It is known that histone H3 has Cys residues in the C-terminal part of the molecule (von Holt *et al.,* 1987) that may form intermolecular bridges with other proteins. Purified virus as well as cellular and nuclear homogenates prepared from infected and noninfected cells were therefore electrophoresed under nonreducing conditions and were then tested for M1 binding. In the presence and the absence of DTT only the 32- and 14-kDa polypeptides were recognized by M1 in homogenates prepared from both noninfected and infected cells (data not shown). The proteins of purified virus were not recognized either, irrespective of DTT treatment (data not shown). These observations indicate that intra- and intermolecular S-S linkages are not important for the interaction of M1 with other proteins. Finally, we studied the influence of pH on M1–histone complexing. Cellular polypeptides were transblotted onto membranes followed by incubation with M1 at pH ranging from 5.2 to 8.4 (Fig. 5). M1 binding occurred between pH 6.4 and 8.4. Some increase of M1 binding to the 32 kD component was observed at pH 8.4, whereas interaction with the 14-kDa components was optimal at neutral pH. The observations that interaction of M1 with histones de-

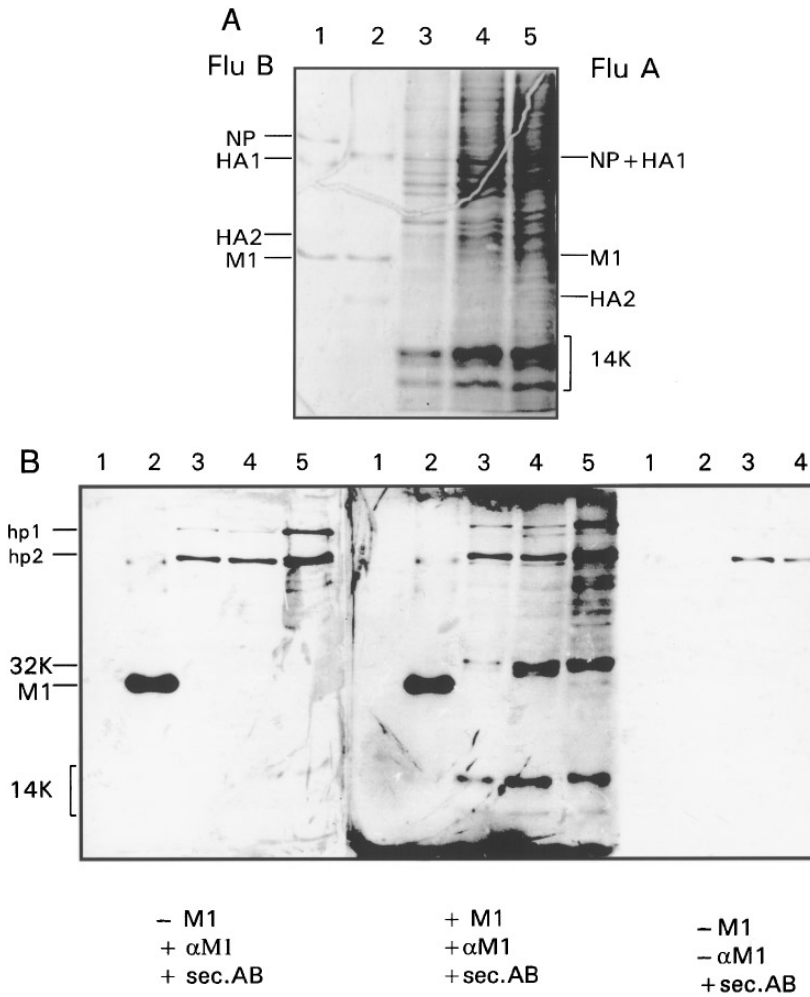

FIG. 2. Cellular polypeptides recognized by M1. Proteins of purified virus and uninfected cells were analyzed by SDS–PAGE on 10% gels: B/HK/72 virus (lane 1), A/Aichi/68 virus (lane 2), MDCK/2 cells (lane 3), MDCK cell (standard line) (lane 4), CV-1 cells (lane 5). (A) The SDS–PAGE gel was stained with Coomassie blue R-350. (B) Polypeptides were blotted onto nitrocellulose membrane and incubated for 1 hr at 37° in buffer, containing either 5% BSA, 100 m*M* NaCl, 100 m*M* Tris–HCl (pH 8.4) or additionally ~0.5 μg/ml of the purified M1 protein. After the incubation, the membranes were exposed successively (as shown on the figure) to anti-M1-monospecific rabbit serum, biotin-conjugated secondary antibodies, and streptavidin–peroxidase complex followed by development with ECL reagents.

pends on salt concentration and that it is inhibited at acidic pH implies the prevalence of ionic forces in the M1–histone complexes.

To extend the observations obtained with membrane-binding assays M1–histone complexing was also analyzed in immune coprecipitation studies. Histones and purified M1 were mixed in solution, and after precipitation of M1 with anti-M1 monoclonal antibodies the presence of histones in the precipitate was analyzed by the Western blot technique with α-His and α-M1 antibodies as probes. As can be seen in Fig. 6 histones comigrating in the 14-kDa band (α-His; lanes 2, 3) and M1 (α-M1; lanes 2, 3) were clearly observed in the immune precipitates displaying the formation of M1–histone heterocomplexes. About 10–25% of histones were precipitated by M1, as indicated by SDS–PAGE and ECL detection experiments in which 5 μg of M1 was added to 20 μg of an equimolar mixture of all five histones. These results show that M1 also interacts with histones effectively in solution.

We also obtained evidence that the amounts of histones coprecipitating with M1 depend on the conformational state of M1. In particular, a brief (1–2 min) pretreatment of purified M1 with high concentrations of NaCl was found to increase its ability to precipitate histones (Fig. 6; α-His; lanes 2 and 3). This observation implies that salt treatment alters the conformation of M1 in a way that facilitates its histone-binding features. Furthermore, in "upside down" membrane-binding assays where M1 was first electrophoresed with SDS, blotted to the membrane, and then covered with histone solution, histones failed to interact with the membrane-bound M1 (data not shown). This finding also supports the conclusion that the conformation profile of M1 is important for the recognition of histones.

In the next set of experiments the involvement of the

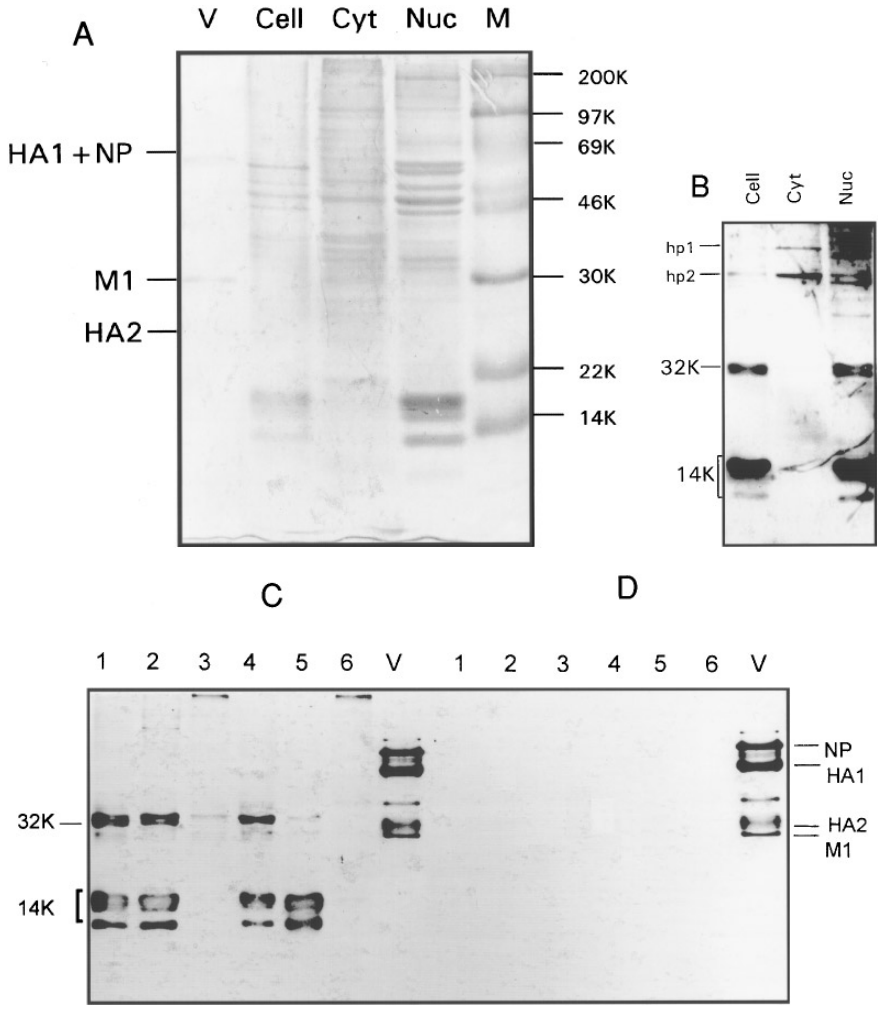

FIG. 3. Subcellular distribution of proteins recognized by M1. Cell homogates (lanes 1, 4), nuclei (lanes 2, 5), and cytoplasmic fractions (lanes 3, 6) from MDCK (lanes 1–3) and CV-1 cells (lanes 4–6) were analyzed by SDS–PAGE. After electrophoresis polypeptides were stained with Coomassie blue R-350 (A) or blotted onto nitrocellulose membranes and incubated in buffer containing 0.5 μg/ml of purified M1 protein of A/Aichi (B) or B/HK (C). After the incubation, the membranes were first exposed to anti-A/Aichi-M1 antiserum (B) or anti-BIHK-virion antiserum (C), then to biotin-conjugated B or HRP-conjugated (C) secondary antibodies, and finally to the streptavidin–peroxidase complex (B). (D) The blot was not exposed to B/HK-M1. Lanes V, M, purified A/Aichi/68 or B/HK/72 viruses and marker polypeptides.

N- and C-terminal parts of M1 in histone interaction has been evaluated. Purified M1 was cleaved with formic acid into 9- 18-kDa fragments (Fig. 7A, lane 3). After such treatment more than 90% of M1 was cleaved, as indicated by scanning of Coomassie-stained gels. Using monoclonal antibodies specific to the N-terminus (clone 1C-6) and the C-terminus (clones 5C-9 and 7E-5) of M1 it was confirmed by quantitative analysis of immunostained blots that the 9- and 18-kDa fragments are the N- and C-terminal parts of M1, respectively (Fig. 7B). This is in a good agreement with previous observations made by Ye *et al.* (1987, 1989). "Upside down" assays in which cleaved M1 were blotted onto PVDF membranes followed by covering with histone solution showed that both fragments failed to bind histones (data not shown). This result is compatible with the concept that correct conformation of the whole M1 molecule is necessary for the interaction with histones.

A 17-kDa polypeptide (referred to as M3) of unknown nature was detected in the specimen of purified M1 by Mab M2-1C6 specific for the N-terminus of M1 (Fig. 7B; lane 1). This M1-related protein was, in contrast to the 18-kDa C-terminal M1 fragment, sensitive to formic acid cleavage and was not recognized by anti-M1 Mabs (clones 7E-5, 5C-9, 904-6) specific to the central and the C-terminal parts (epitopes 2–4) (Ye *et al.,* 1989) of M1 (Fig. 7B). These observations exclude the trivial explanation that M3 protein is a simple proteolytic cleavage product of M1. The M3 protein was also found as a minor component in purified preparations of all viruses studied, A/WSN/33, A/FPV/Rostock, A/Aichi/68 (data not shown). Further investigation of the M3 protein was not undertaken in his study. A 15-kDa polypeptide observed by Ye *et al.* (1987) in M1 preparations purified from WSN virus seems to be a similar M1 product.

Further, the histone-binding capacity of noncleaved

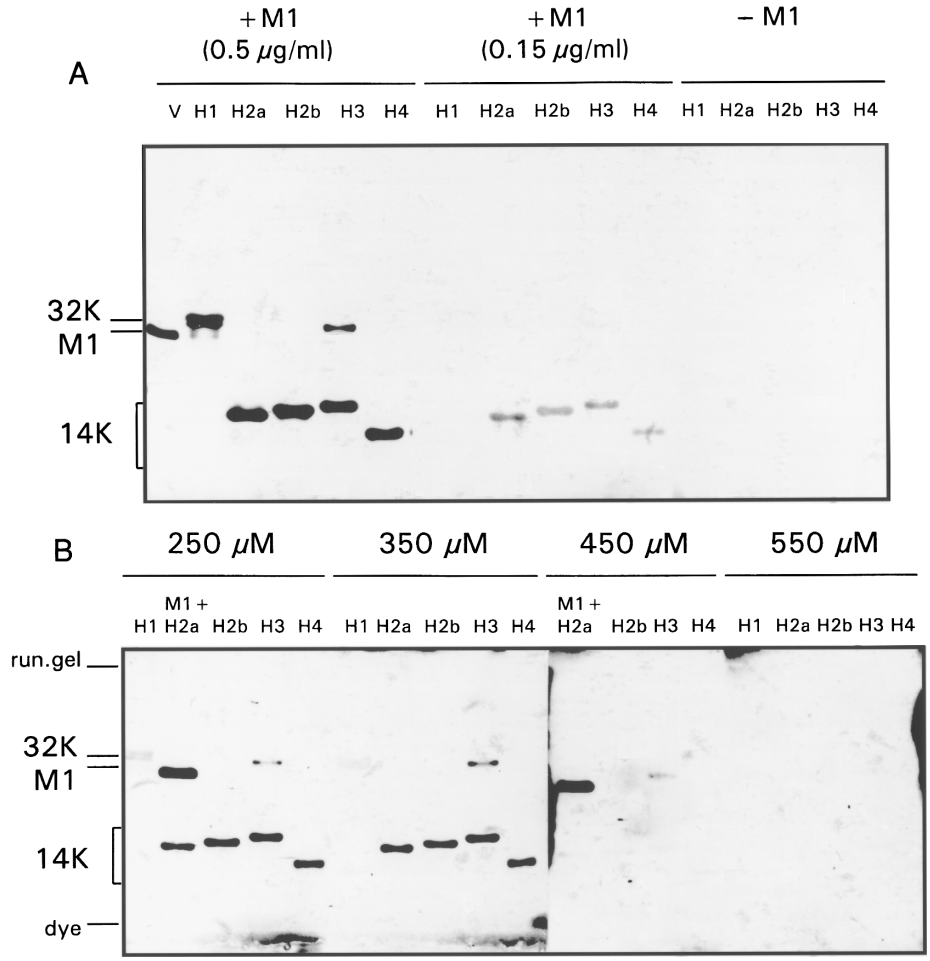

FIG. 4. Western blot analysis of M1 interaction with purified histones. (A) Histone polypeptides H1, H2A, H2B, H3, H4 isolated from calf thymus (Boehringer Mannheim) were analyzed by SDS–PAGE on 12% gels with ~1 μg of each histone per gel lane. The proteins were then transferred onto a nitrocellulose membrane and incubated in buffer either containing 0.5 or 0.15 μg/ml Aichi M1 or lacking M1. Membranes were then incubated with monoclonal anti-M1 Ab (Serotec) for 1 hr at 20° and processed using secondary Ab and the ECL detection system. Lane V: purified A/Aichi/68 virus. (B) Histone polypeptides were processed as described in (A), except that membranes were incubated with M1 in buffer containing 250, 350, 450, and 550 m*M* NaCl. As an internal M1-positive control, purified Aichi/68 virus was added to the sample of H2A histone before electrophoresis as indicated.

and cleaved M1 was compared by direct membrane-binding assays. The following results were obtained (Fig. 7C). (i) Both noncleaved and cleaved M1 interact with histones (lanes 1, 3, 4). (ii) In the case of cleaved M1, both N- (9K) and C-terminal (18K) polypeptides bound to histones and were recognized in histone–M1 complexes by anti-M1 Mabs specific to the N- and C-terminal domains (lanes 3, 4). (iii) In agreement with the above coprecipitation experiments (Fig. 6; α-His; lanes 2, 3), salt pretreatment of noncleaved M1 increases binding to histones (Fig. 7C; lanes 1 and 2). (iv) The cleaved M1 pretreated with high salt loses the ability to bind to histones (Fig. 7C; lanes 5, 6). These findings additionally show that the tertiary structure of M1 is an important factor in the interaction with histones and that both N- and C-terminal parts of M1 are involved in complexing.

DISCUSSION

The data obtained have shown that protein M1 isolated from influenza virions by acidic extraction has the ability to bind to histone proteins H2A, H2B, H3, and H4 and more weakly to H1. The M1–histone interaction was not dependent on bivalent cations and EDTA; however, it was prevented by acidic pH and high salt concentrations. These findings suggest that ionic forces are prevalent in M1–histone complexes. The interaction depends on the conformation of M1. Alteration of salt bridges in M1 was found to significantly increase binding to histones. Since salt links are known to stabilize the tertiary and quaternary structures of proteins it is tempting to speculate that histone-binding sites are cryptic and have to be exposed before binding can occur. This notion is also compatible with the view that M1 is a typical allosteric protein with different active conformations responsible for virion assembly, RNP transport, nuclear histone binding, and membrane binding.

A striking feature of histones is their high content of positively charged side chains: about 25% of the amino acids are lysine and arginine (Grunstain, 1990; von Holt

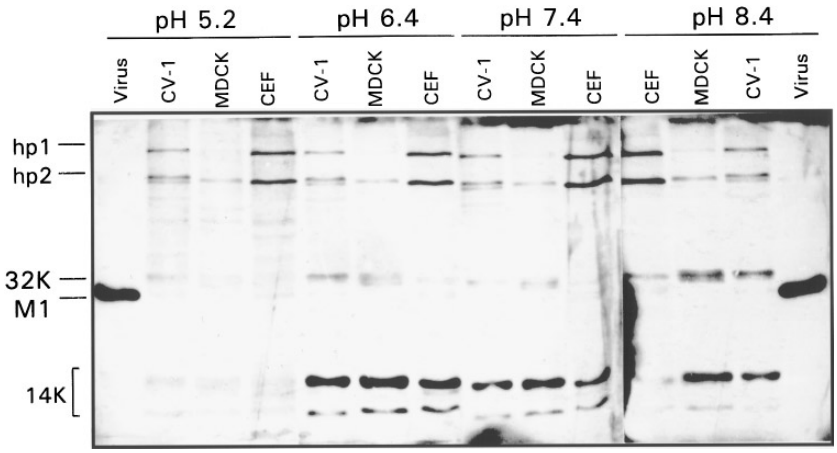

FIG. 5. pH-dependent binding of M1 to cellular polypeptides. Homogenates of CV-1, MDCK, and CEF cells were electrophoresed and then transferred onto nitrocellulose membranes. The membranes were incubated with M1 (0.5 μg/ml) in 0.1 *M* Tris–Mes buffer at pH 5.2, 6.4, 7.4, or 8.6 and decorated using anti-M1 antibodies and the ECL detection system. Lane V: purified Aichi/68 virus.

et al., 1987). These positively charged amino acids are concentrated preferentially at the N-termini of histones H2A H2B, H3, and H4 and at the C-terminus in H1 histone (Vettese-Dadey *et al.,* 1994; von Holt *et al.,* 1987). Influenza virus matrix protein M1 is also a highly basic polypeptide, with a p*I* of about 8.8 (Leavitt *et al.,* 1979). As can be seen from the sequence data (Briedis *et al.,* 1982; Yamashita *et al.,* 1988), a peculiarity of M1 consists in a preferential distribution of basic amino acids in the central and the C-terminal part of the molecule, whereas its N-terminus is rich in glutamic and aspartic acids residues. On the basis of these data it is reasonable to assume that M1–histone complexing is mediated by the interaction of the differentially charged N-termini. This idea is supported by the observation that exposure to acidic pH, making the Glu and Asp residues of the M1 N-terminus noncharged, prevents binding of M1 to histones. The N-terminal domain alone, at least in the context of the 89 N-terminal amino acids forming the 9-kDa cleavage polypeptide, did not bind to histones. It therefore appears that the histone-binding site of M1 is a conformational one that requires a specific tertiary and possibly quaternary structure for activity.

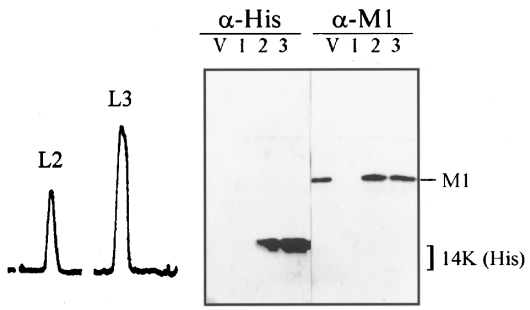

FIG. 6. Immune coprecipitation analysis of M1–histone complexing. Histone mixtures (Boehringer) were added to PBS (lane 1), purified FPV M1 (lane 2), or M1 pretreated with 1 *M* NaCl (lane 3). After 1 hr incubation anti-M1 Mab M2-1c6 was added, and immune complexes were absorbed to protein A–Sepharose. Protein A-absorbed polypeptides were dissolved in SDS–DTT buffer, and aliquots were analyzed in parallel on 13.5% polyacrylamide gels. Lane V: electrophoresis of purified FPV virus polypeptides. Each of two gel parts was visualized by Western blot–ECL with either sheep anti-histone or goat anti-M1-monospecific sera. Scanning profiles of the 14-kDa histone bands from lanes 2 and 3 are shown on the left.

M1–histone complexing appears to be a specific interaction. First, M1 selectively recognizes histones in homogenates of infected and noninfected cells and in the nuclear fractions. Second, M1–histone complexing is characterized by high affinity in quantitative terms; 150–250 ng of histone used for transblots is sufficient for binding detectable amounts of M1 from incubation medium containing as low as ~150 ng ml of M1.

The functional significance of M1 binding to histone proteins remains unclear. It is known that newly synthesized M1 enters the nucleus of infected cells. In the nucleus, M1 seems to be required for the export of vRNP from the nucleus to the cytoplasm. On the basis of observations indicating that newly synthesized M1 and NP proteins colocolized both in the nucleus and in the cytoplasm of influenza virus-infected cells it has been suggested that M1 associates with newly assembled vRNPs and escorts their nuclear exit (Martin and Helenius, 1991b). On the other hand, studies performed on an influenza virus with a temperature-sensitive mutation of M1 (Sugiura *et al.,* 1975) have shown that nuclear export of vRNP was not altered, although M1 was retained in the nucleus at nonpermissive temperature (Rey and Nayak, 1992). It has therefore been suggested in this study that exit of M1 into the cytoplasm is not needed for nuclear export of vRNP (Rey and Nayak, 1992). Recent data, obtained in a heterokaryon system (Whittaker *et al.,* 1996) and with the M1 hyperphosphorylation mutant of WSN virus (Whittaker *et al.,* 1995), support this point of view.

Based on the observations described here we propose the following working hypothesis. First, the vRNP transcriptive/replicative complex functions in the nucleus in close contact with active (decondensed) chromatin struc-

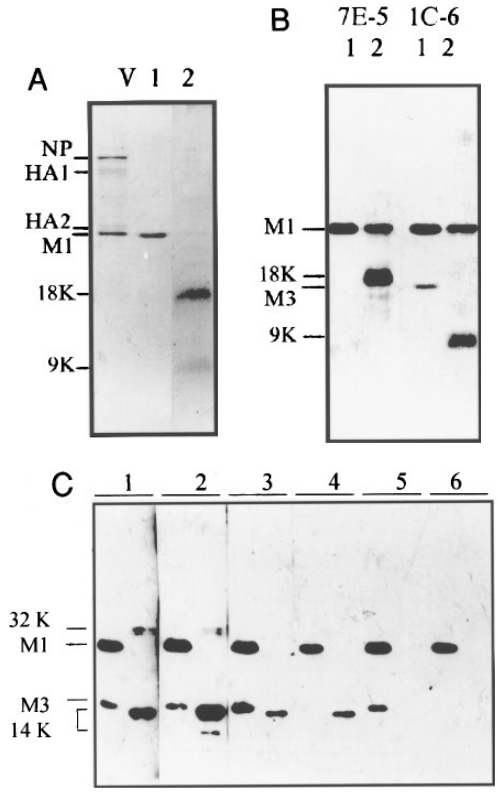

FIG. 7. Interaction of cleaved M1 with histones. Purified Aichi M1 was cleaved with formic acid. Noncleaved (lane 1) and cleaved (lane 2) M1 were subjected to SDS–PAGE and then to either staining with Coomassie blue R-350 (A) or Western blot analysis with ECL visualization using 1C-6 and 7E-5 anti-M1 MABs (B). Purified Aichi virus, as a positive internal control, (left lanes) and a mixture of histones H1, H2A, H2B, H3, and H4 (right lanes) were electrophoresed in SDS–PAGE and then blotted onto 0.22-μm PVDF membrane (Bio-Rad) (C). The membrane was cut into strips and incubated in buffer containing 0.5 μg/ml nontreated (lane 1) or 1 *M* salt-treated (lane 2) Aichi M1 and $\sim$0.6 μg/ml nontreated (lanes 3, 4) or 1 *M* salt-treated (lanes 5, 6) Aichi M1 cleaved by formic acid. After 1 hr incubation at 20°, the membranes were visualized by the ECL technique using either Mab 1C-6 specific to the N-treminal part of M1 (lanes 1–3, 5) or Mab 7E-5 specific to the C-terminal part of M1 (lanes 4, 6) and anti-mouse HRP-conjugated Ig.

tures. These decodensed regions (20–40 per nucleus) seem to be involved in the synthesis of host cell mRNAs (Carter *et al.,* 1993), and such capped mRNA may be used by the viral polymerase complex to initiate the synthesis of viral mRNA (Krug *et al.,* 1979). Furthermore, the association of vRNP with chromatin (Bukrinskaya *et al.,* 1979; Hudson *et al.,* 1978), nuclear matrix (Jackson *et al.,* 1982; Lopez-Turiso *et al.,* 1990), and M1 protein (Patterson *et al.,* 1988) was reported to take place in the nucleus of influenza virus-infected cells. M1 is known to be a late viral protein since its synthesis starts in infected cells at 5–6 h.p.i. (Inglis *et al.,* 1976). At this stage of the infection cycle, newly synthesised M1 may enter the nucleus and recognize the vRNP-decondensed chromatin complexes. By binding to histones in the chromatin nucleosome, M1 may push out the vRNP from the complexes and prevent the association of newly synthesized vRNP with chromatin promoting the exit of vRNP into the cytoplasm. Certainly, further studies are needed to prove this hypothesis. Second, it is known that two copies each of histones H2A, H2B, H3, and H4 assemble into an octameric core around which a helical DNA is wrapped, forming a nucleosome. The fifth histone, H1, is bound to DNA in the interface as linker between nucleosomes (Grunstain, 1990). The Arg/Lys-rich amino termini of the histone proteins extend outward from the nucleosome, thus facilitating their recognition and interaction with M1. Numerous histone interacting nuclear transcription regulatory polypeptides (Coleman, 1992), such as TFIII-A (Laybourn and Kadonaga, 1991) and GAL4-AH (Vettese-Dadey *et al.,* 1994), contain both histone binding and Zn-finger motifs. Like these proteins, M1 has a Zn-finger sequence (Elster *et al.,* 1994; Wakefield and Brownlee, 1989), which is required for virus replication (Nasser *et al.,* 1996). It is tempting to speculate that the Zn-finger motif of M1 is involved in an interaction with host cell chromatin in virus replication.

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